International Journal of Biological Research, 4 (2) (2016) 249-259

International Journal of Biological Research

Website: www.sciencepubco.com/index.php/IJBR
doi: 10.14419/ijbr.v4i2.6755
Research paper

Differential toxicity of Pb & Hg on the development of modular traits, photosynthetic and biochemical attributes in two varieties of a forage crop species *Trifolium alexandrinum* L.

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Abstract

Heavy metal stress as result of natural and anthropogenic activities is main environmental problem. Pb and Hg are among non-biodegradable metals thus remaining persistent in soil and water. The present study was carried out to assess growth and biochemical responses of two varieties (Desi and Misri) of *Trifolium alexandrinum* L. after application of varying levels (25mg/kg, 50mg/kg and 100mg/kg) of Pb and Hg in soil along with control. Seed germination, biomass of above and below ground tissues, number of flowers and leaves, leaf area and nodulation was observed. For biochemical attributes, green pigments, protein and amino acids, were determined. Both varieties (Desi and Misri) showed variable responses in relation to both Pb and Hg. Similarly, the pattern of character expression was independent for metal levels and types. Misri performed consistently better as it showed best threshold for most of the attributes studied. Hg was found to be more toxic as compared to the Pb as it induced more drastic decline in parameters studied. The study showed that biometric traits can be used as good predictors and the biochemical parameters cannot be used as useful biochemical markers as they showed no marked disparity.

Keywords: *Biochemical Markers; Biometric Traits; Desi; Misri; Trifolium Alexandrinum.*

1. Introduction

Heavy metal pollution is an international increasing environmental dilemma. The cultivated lands polluted with heavy metals due to anthropogenic activities are of serious concern [1, 2]. Supra optimal concentrations of both essential and non-essential metal ions are phytotoxic (Hamid et al., 2010). However, the responses of plants vary widely depending on their species and types of metals (Tomas et al., 2012).

Like many other developing countries, tremendous industrialization in Pakistan had resulted in disposal of industrial waste containing heavy metals into water bodies. The contamination of water bodies poses threats to both aquatic and terrestrial life forms (Marshall et al., 2007). In addition, the use of contaminated water for irrigation purposes can cause long-lasting metal contamination of agriculture soils (Singh et al., 2004; Sharma et al., 2007). Moreover, the use of municipal waste as fertilizer owing to high cost and shortage of fertilizers also resulted in metal pollution because the waste is heavily loaded with heavy metals such as Cu, Zn, Pb and Hg (Mushtaq and Khan, 2010). Pb and Hg are added to the environment as a result of both natural and anthropogenic activities. An incredibly use of automobiles is a major source of Pb pollution. Similarly, substantial amounts of mercury are introduced in the form of fertilizers and pesticides (Selin, 2009; Khan et al., 2011; Walter et al., 2011; Jalaluddin & Hamid, 2012).

Pb and Hg pollution is of great concern because both of these metals are non-essential and non-biodegradable thus persists indefinitely in soil and water. Despite their non-essentiality, plants have a tendency to uptake and translocate them within their tissues when metal exists in higher concentrations (Elavrasi and Dhanam, 2012). Although metal tolerant species have shown a better thresh for Pb and mercury but exceeding amount of metal present in the plant tissues is phytotoxic. Metal toxicity is associated with visible damage to plants (Islam et al., 2008; Liu et al., 2010) as well as metabolic disorders which includes inactivation of enzymes, photosynthesis, respiration, chlorophyll biosynthesis, oxidative stress and inhibition of DNA synthesis thus disturbance of mitosis (Rossato et al., 2012).

Egyptian clover (*Trifolium alexandrinum L.*) is an important green fodder. Due to the highest fodder yield (100-125t ha^{-1}) it is also called as the ‘king of fodder’ and it is able to give 5-7 cuts of succulent forages (Khalil and Jan, 2000; Mahmood and Abbas, 2003). In addition, its high palatability, digestibility (about 65%) and protein enriched containing 20% crude protein (Malaviya et al., 2004).

In Pakistan, forage crops are cultivated in over 10.3% of the total cultivated area of 22.6 million hectares. Thus, production of fodder in the country is low to support livestock including small ruminants (Agricultural Statistics of Pakistan 2009-10) and is the main hindrance for livestock and dairy development.

Despite considerable significance of forage crops in the country, there has been given little attention to develop or improve forage crops which can be grown under a wide array of environmental situations and constraints. Therefore, it is imperative that intensive forage cultivation per unit area per season should be practiced (Dost et al., 1990, Bhatti et al., 1992) by exploiting high yielding varieties for metal polluted agricultural lands.

Keeping in view the above aspects, two local varieties of *Trifolium alexandrinum* L. were assessed for their performance under Pb and Hg pollution. The modular traits development, photosynthetic and biochemical attributes of the varieties were assessed to appraise their potential for metal contaminated soils of the country.

2. Materials and methods

The seeds of two varieties of *Trifolium alexandrinum* L. namely Desi and Misri were obtained from local market of Multan. The experiment was conducted under natural conditions during January-March, 2012 at Botanic Gardens, Bahauddin Zakariya University Multan, Pakistan. The sources of metals used were their respective salts i.e. $Pb(NO_3)_2$ and $HgCl_2$.
Germination experiment was performed in a laboratory under natural conditions and at room temperature (± 25 ºC). Plastic germination trays were used for this purpose having 72 equal sized cells (each cell had diameter of 4cm). The hole below each cell was blocked using a water proof self-adhesive plastic tape. Sand was sieved through a 2mm sieve and washed with tap water to clear all the dirt and then air dried. Both salts $(PbNO_3)_2$ and $(HgCl_2)$ were completely mixed with sand in a ratio of 25, 50 and 100mg/kg.
Total forty eight appropriately labeled cells were randomly filled with sand of different levels of both salts. There were made three replicates for each treatment. Thus each variety had a set of twenty four cells, twelve cells for each salt. Each cell was filled with 45g sand. Five seeds of each variety namely Desi and Misri were sown in each cell according to the following treatments for both $Pb(NO_3)_2$ and $HgCl_2$ i.e., T_0-Control (sand), T_1-25mg/kg, T_2-50mg/kg and T_3-100mg/kg. Germination records were made through emergence data and data were presented as germination percentage using the following formula:

$$\text{Germination Percentage} = \frac{\text{Number of Seeds Germinated}}{\text{Total Number of Seeds Sown}} \times 100$$

After germination experiment, seeds were sown in earthen pots. For this compost soil was used. The soil was sieved through 2mm sieve. Forty earthen pots (length 15cm and width 16cm) were used and tagged for both salts and varieties. The hole at the base of each pot was blocked completely with muslin cloth to avoid leaching. Lead Nitrate ($Pb(NO_3)_2$) and Mercury Chloride ($HgCl_2$) were thoroughly mixed in compost soil.
Three replicates were made for each treatment. Each variety had a set of twenty four pots, twelve for each salt. The experiment was organized in a Complete Randomized Manner. Five seeds of both (Desi and Misri) varieties were sown in each pot according to the following levels of both salts ($Pb(NO_3)_2$ and $HgCl_2$) i.e., T_0-Control (sand), T_1-25mg/kg, T_2-50mg/kg and T_3-100mg/kg. The plants were watered daily. After 16 weeks, the plants were harvested by taking out the whole plant from the pot. The soil was removed from the roots and washed with water to remove extra dirt. Then roots were separated from their respective shoots and were placed in labeled paper bags for further analysis.
Different biometric and biochemical parameters were studied. The root and shoot length of each plant were determined by inch tape. The trifoliate leaf from each replicate of all the levels of both metals and varieties was taken and its area was determined by scanning and taking print on centimeter graded graph paper. Fresh and dry biomass of roots and shoots were determined by a digital balance. The number of leaves, flowers and root nodules were calculated of each plant. The contents of pigments were determined using Arnon (1949) method. Fresh leaves from each sample about 0.2g were cut and grinded in pestle and mortar with 4ml of 80% acetone. The extracts were filtered and their respective filtrates were read at 645, 663 and 480nm using a spectrophotometer (Biotechnology Medical Services, UV-1900, USA).
The chlorophylls a, b and total were determined by the following formulae:
Chlorophyll a (mg/g) = (12.7 (O.D 663) – 2.69 (O.D 645)) x V/1000 x W

Chlorophyll b (mg/g) = (22.9 (O.D 645) – 4.68 (O.D 663)) x V/1000 x W

Total Chlorophyll (mg/g) = 20.2 (O.D 645) + 8.02 (O.D 663) x V/1000 x W

Kirk and Allen (1965) formula was used to calculate carotenoids:

Carotenoids (mg/g) = O.D 480 + 0.114 (O.D 663) – 0.638 (O.D 645)

Total free amino acids were determined by Hamilton and Van Slyke (1943) method. 1ml of extract was mixed with 1ml of 10% pyridine and 1ml of 2% ninhydrin solution in 25ml test tubes. The tubes were heated in boiling water bath for 30 min and allowed to cool at room temperature. The volume of the mixture was made up to 50 ml with distilled water and the OD of the extract was read at 570 nm using a spectrophotometer (Biotechnology Medical Services, UV-1900, USA). A standard curve was developed with Leucine and frees amino acids were calculated. Total protein content was determined by Bradford (1976) method. Optical densities were read at 595nm. A standard curve was made with the Bovine Serum Albumin and the protein contents were calculated.
The data was then subjected to ANOVA General Linear Model by using Minitab 16 statistical software, to determine significant influences of varying levels of each metal on both varieties. The parameters having significant F ratios were subjected to Duncan Multiple Range Test (DMRT), (Duncan, 1955).

3. Results and discussion

The seed germination is the primary criteria to monitor effect of pollution (Shanmugavel, 1993) because many environmental pollutants can affect seed germination which is the first developmental stage in the life cycle of a plant. Metal pollution appeared to cause a noticeable reduction in germination. Although, the varieties (Desi and Misri) depicted contrasting response in their emergence when exposed to varying levels of lead and mercury. The germination of Desi was less influenced by lead and mercury whereas the emergence of Misri was more in response to mercury only. Any alteration in the soil environment can influence optimal conditions which are crucially important for the successful establishment of plants and consequently the production of biomass or yield (Elavrasi and Dhanam, 2012). Increasing pollution dosages are well known to cause reduction in viability of seeds at different rates and the sensitivity also varied in different plants (Chaphekar, 1991).
For root length, the two varieties; Desi and Misri had similar response to both metals as consistently longer roots were observed in response to Pb than Hg. Similar decline in root length was also reported in many other species. For example, in wheat (Datta et al., 2009; Mesmar and Jaber, 1991), Albizia lebbeck (Farooqi et al., 2009) and *Brassica juncea* (Donghua et al., 2000). The greater influence of Pb on roots can be attributed to its accumulation in the roots due to less mobility to aerial tissues. It has been well reported that Pb in higher concentrations can cause reduction in mitotic division in meristematic region of root of *Allium cepa* (Lerda, 1992) therefore less root elongation (Goldbold and Kettner, 1991; Sharifah and Hishashi, 1992). Same results have been reported by Peralta et al., (2001). Although, root growth inhibition by mercury has been reported but greater accumulation of mercury in the roots and it's binding in cell wall may result in less toxicity (Wang and Greger, 2004). The results of this study are also parallel to these findings as root elongation was not much influenced by Hg.
There has been observed no contrasting responses of the varieties for shoot length as both Desi and Misri varieties showed no interspecific disparity for both metals. Shoot length of both varieties was not influenced by Pb levels. The lesser toxicity of Pb might be due to its restricted penetration from roots to aerial parts of the

plants. According to Sharma and Dubey, (2005), the root endodermis in maize and barley acts like a barrier for Pb uptake by the shoots. However, by contrast Hg had caused decline in shoot length though was less injurious to the roots. Thus it appears that endodermis can become a week barrier against elevated level of Hg. Similar Hg-induced stunted growth has been reported by many workers in many plants (Cho and Park, 2000; Cargnelutti et al., 2006; Ge et al., 2009; Costa et al., 2011). Mercury causes ultra-structural damage that has inhibitory effect on plants growth (Israr et al., 2006; Zhou et al., 2008; Shiyab et al., 2009a).

Plant growth and performance under heavy metal stress is frequently predicted via fresh and dry biomass production which may serve as an important indicator of plant success under stressful environment. Since Trifolium is a multiply harvested crop therefore vegetative growth can be assessed through biomass of plants. The below ground tissues growth can be adversely affected due to heavy metal ions as roots are the first organs that come in contact with contaminated soil. The above ground parts of plants are important as they support and accumulate photosynthetic products. Both fresh and dry biomass of root in varieties did not vary greatly as both varieties had more biomass in response to Pb than Hg. The increasing trends for root and shoot biomass are in close agreement to Wierzbicka, (1998), who reported an increase in the dry weight of organs of corn plant with an increase in Pb content which was due to an increase in the production of cell wall polysaccharides. However, no such trends were observed for Misri thus showed a progressive decrease in biomass with increasing Pb concentrations.

On the other hand, mercury (Hg) induced remarkable decline in the fresh and dry biomass of root and shoot in both Desi and Misri varieties. Thus, Hg appeared to be more injurious than Pb.

Our results showed that both Pb and Hg caused noteworthy decline in number of leaves and flowers in both Desi and Misri varieties. The decline in foliage and flower exhibited a similar trend that was observed for biomass. The decline in leaf number is in in accordance to Kabir et al., (2010) who reported same results for *Thespesia populnea* L. in which increasing Pb dosages caused a decline in foliage. Similarly, Pb at a lower dose is less toxic as compared to the high dose for flowers induction. Khan and Chaudhry, 2006; Tooke et al., 2005 reported that heavy metals stress can delay or stop flowering.

Leaves as light capturing and photosynthetic organs are very vital for the plants. Under Pb toxicity inhibition of leaf expansion has been reported by Gabara and Goaszewska, 1992; Piechalak et al., (2003). Lead induced decline in leaf area has also been stated by Tanyolac et al., (2007). Leaf area was not influenced by Pb in both varieties thus leaf expansion was not influenced remarkably by Pb but Mercury had greatly influenced leaf area of the tested varieties.

Nodulation is a characteristic of leguminous plants through which atmospheric nitrogen is fixed and becomes available to plants. The use of fungicides containing heavy metals have been reported to reduce Rhizobium population and root nodules formation in *Vigna radiata, V. mungo,* and *Arachis hypogaea* (Ghosh, 1995). The extent of nodulation in both Desi and Misri varieties showed a progressive decline in a concentration dependent manner. The presence of heavy metal had affected nodulation in *Vicia faba* L. roots by affecting nitrogenase enzyme or interfering function of leghaemoglobin protein (Beltagi, 2005). Thus reduction in nodule number can also be attributed to above reasons.

Chlorophyll assessment being one of the vital plants attributes and is an index of productive capability of the plant (Li et al., 2007). The inhibition of chlorophyll biosynthesis reduces the total chlorophyll content and comparative content proportion of Chlorophyll a and b (Van Assche and Clijsters, 1990; Sinha et al., 1993; Ernst et al., 2000). Pb inhibits strongly the key enzyme of chlorophyll biosynthesis, δ-amino laevulinate dehydrogenase (Prassad and Prassad, 1987). In the present study metals did not influence chlorophyll a content in both varieties as compared to the control which may be due to the tough structure of chloroplasts (Stiborova et al., 1986; Areu and Lagetta, 1997) that cannot be destroyed. Nevertheless, an overall decrease in chlorophyll is in accordance with other reports (Cho and Park, 2000). Several studies have also shown that persistent Hg exposure affects photosystem II (PS II) by inhibiting the photosynthetic electron transport chain (Bernier et al., 1993; Bernier and Carpentier, 1995).

It is well known that oxidative stress resulted by the heavy metals is by many enzymatic and non-enzymatic anti-oxidants that scavenge oxidative stress. Carotenoids are an important component of non-enzymatic defense. A remarkable increase in carotenoid contents in varieties at elevated levels of both Pb and Hg might be an indicator of a mechanism through which the species might combat heavy metal stress.

It has been reported by Srivastava et al., 2005, that stress proteins that are synthesized after exposure of plants to several abiotic stresses result in elevated protein content. These may be many antioxidant enzymes or heat shock proteins (Prassad, 1996; Srivastava et al., 2005). However, Pb toxicity mostly induces decrease in protein content (Mohan and Hosetti, 1997). Similarly, Hg can affect the proper functioning of enzymes due to protein precipitation that results in loss of defensive mechanism under stressful environment (Patra and Sharma, 2000). Hg exposure to both varieties induced decline in protein content. Desi showed the maximum decline at 100mg/kg of Pb but Misri had the least content of protein at 25 & 50 mg/kg of Hg as compared to the control. Thus, reduction in protein in the studied varieties is parallel to the above findings. Same results were observed in the case of amino acids content that are difficult to explain.

Regarding the overall performance of both varieties to Pb and Hg, Misri variety was more tolerant as it depicted outclass response for ten out of total seventeen parameters. For the rest of seven parameters Desi had better threshold for both Pb and Hg. Hg was found to be more toxic as compared to the Pb for both varieties but Misri variety performed best in this metal toxicity as compared to the Desi variety.

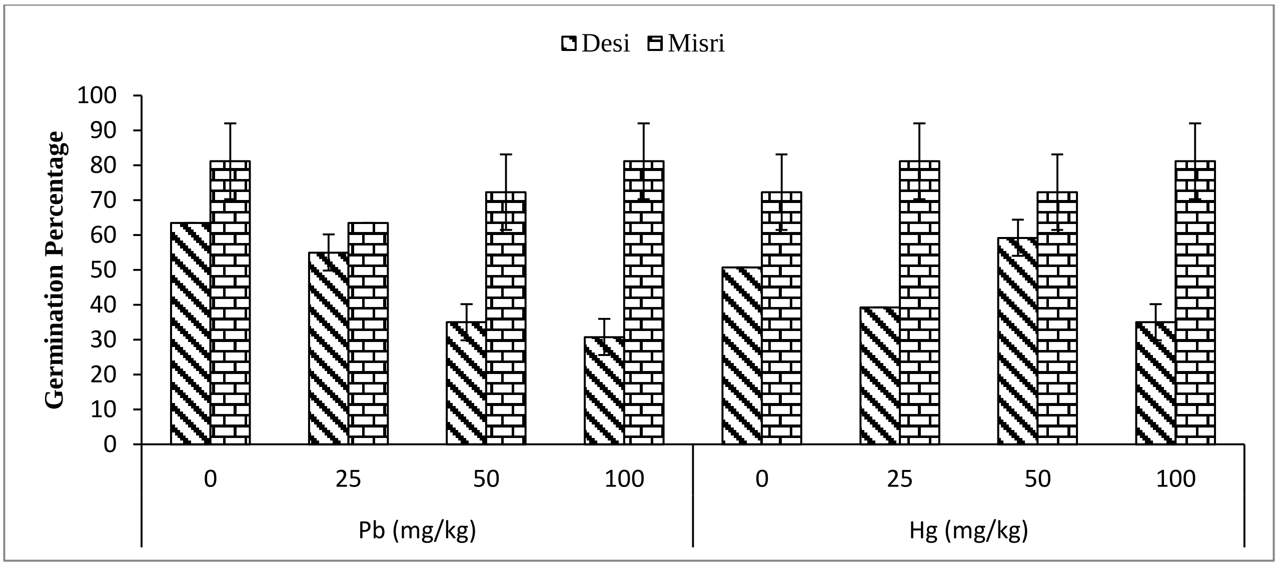

Fig. 1: Germination Percentage of Desi and Misri varieties of *Trifolium alexandrinum* after Exposure to Control and Different Levels of Pb and Hg.

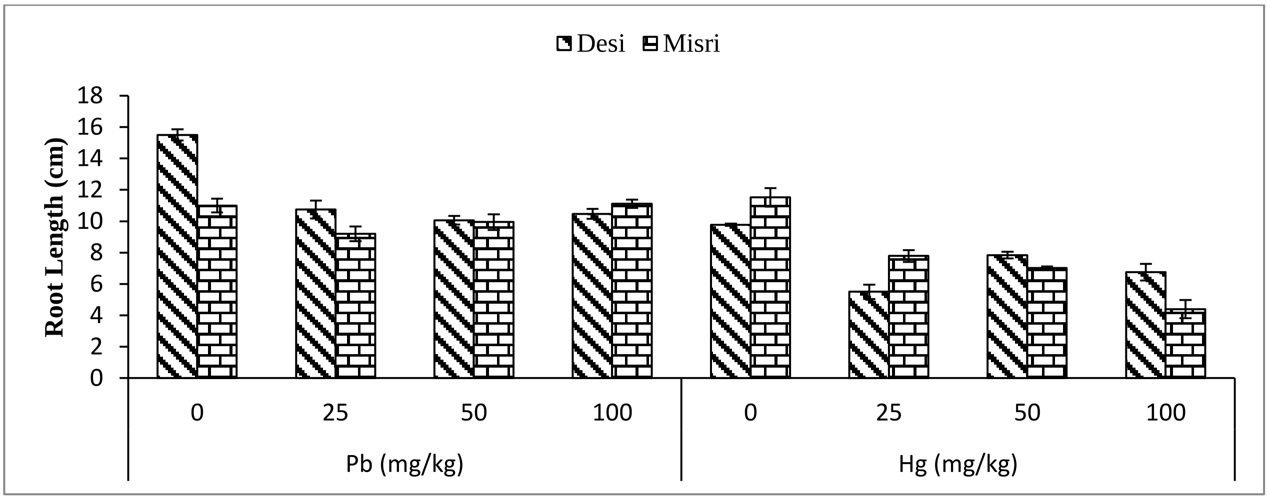

Fig. 2. Root length (cm) of Desi and Misri varieties of *Trifolium alexandrinum* after exposure to control and different levels of Pb and Hg

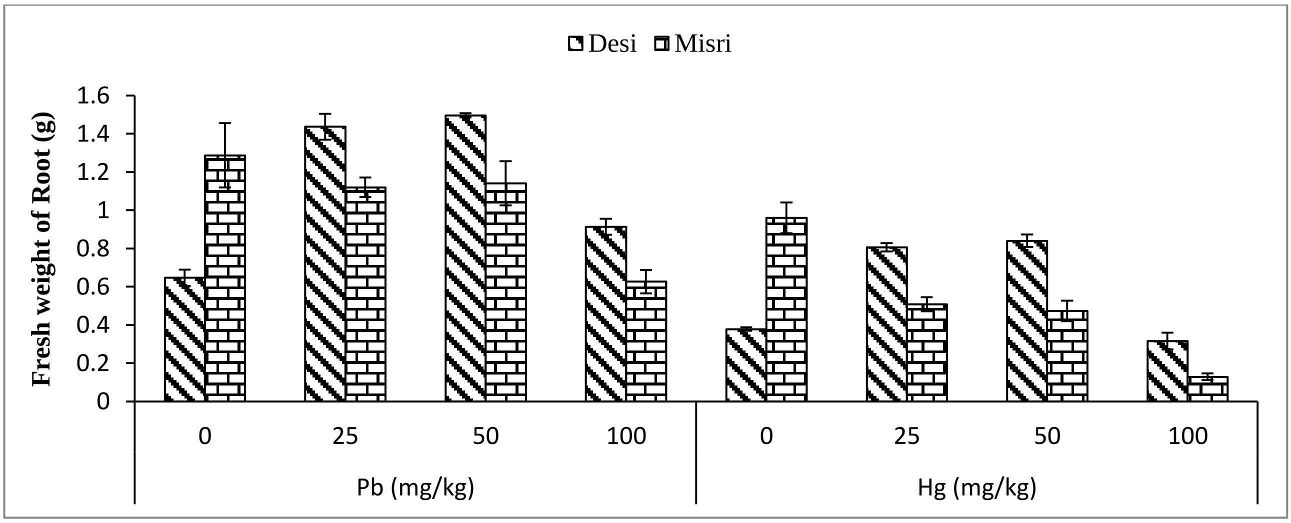

Fig. 3: Fresh Weight of Root (g) Of Desi and Misri varieties of *Trifolium alexandrinum* after Exposure to Control and Different Levels of Pb and Hg.

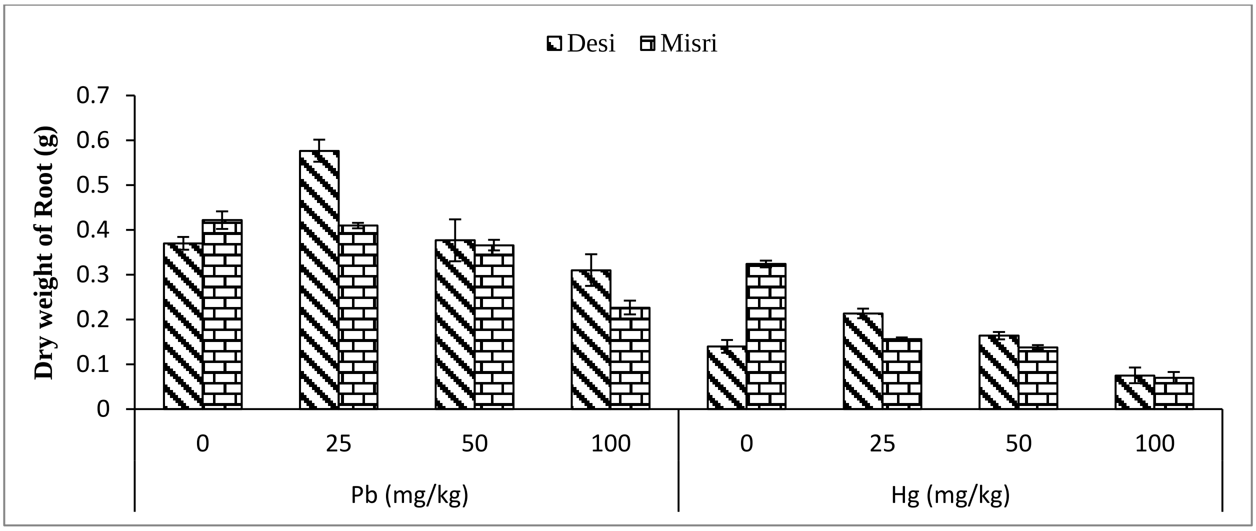

Fig. 4: Dry Weight of Root (g) of Desi and Misri varieties of *Trifolium alexandrinum* after Exposure to Control and Different Levels of Pb and Hg.

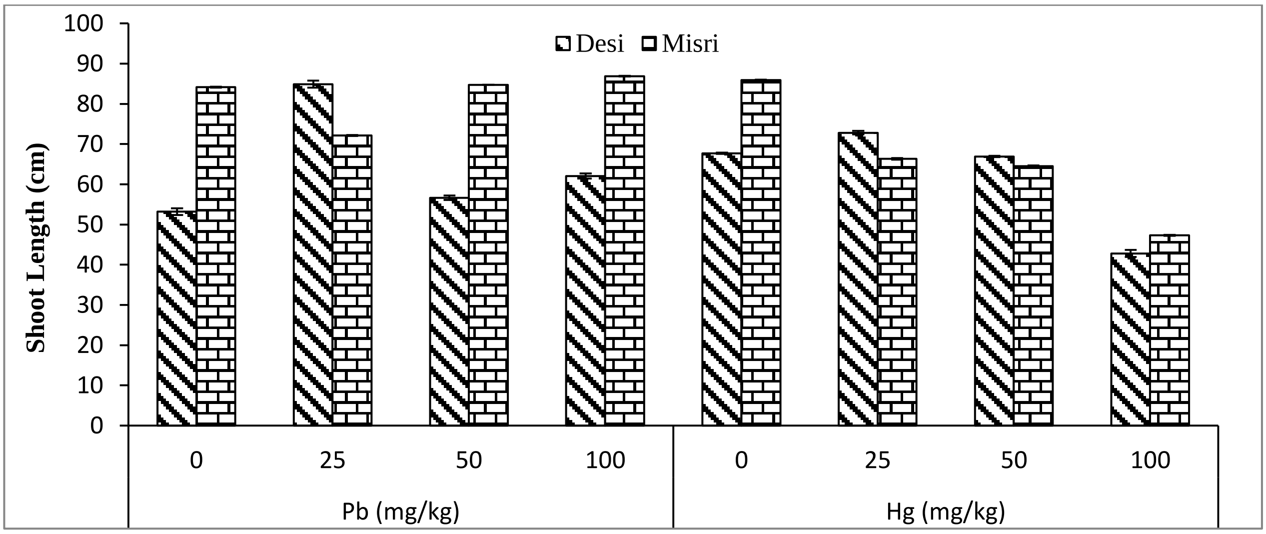

Fig. 5: Shoot Length (cm) of Desi and Misri varieties of *Trifolium alexandrinum* after Exposure to Control and Different Levels of Pb and Hg.

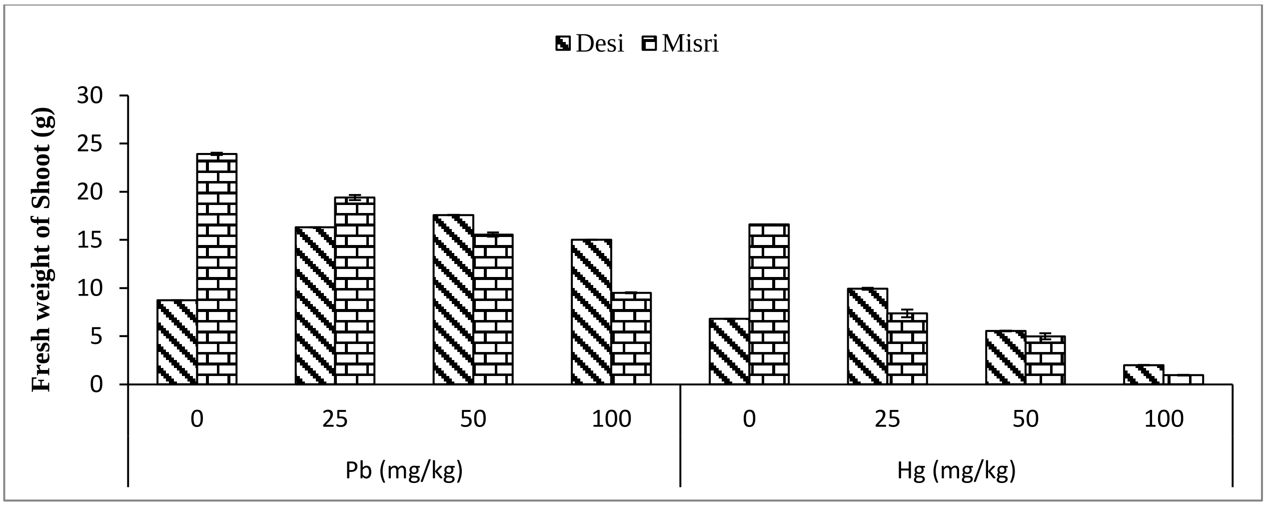

Fig. 6: Fresh Weight of Shoot (g) of Desi and Misri varieties of *Trifolium alexandrinum* after Exposure to Control and Different Levels of Pb and Hg.

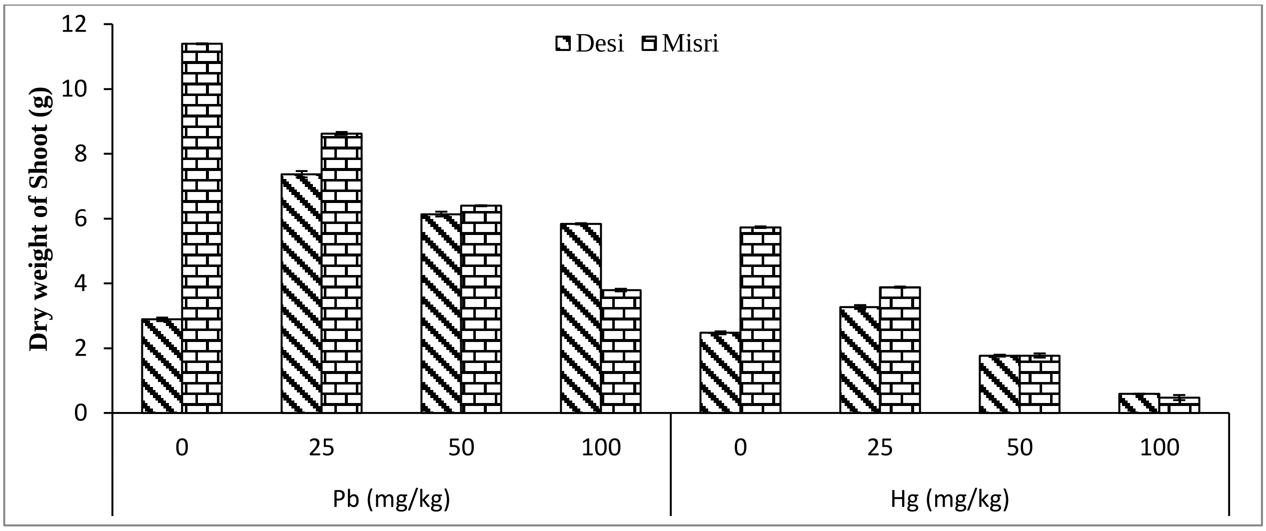

Fig. 7: Dry Weight of Shoot (g) of Desi and Misri Varieties of *Trifolium alexandrinum* after Exposure to Control and Different Levels of Pb and Hg.

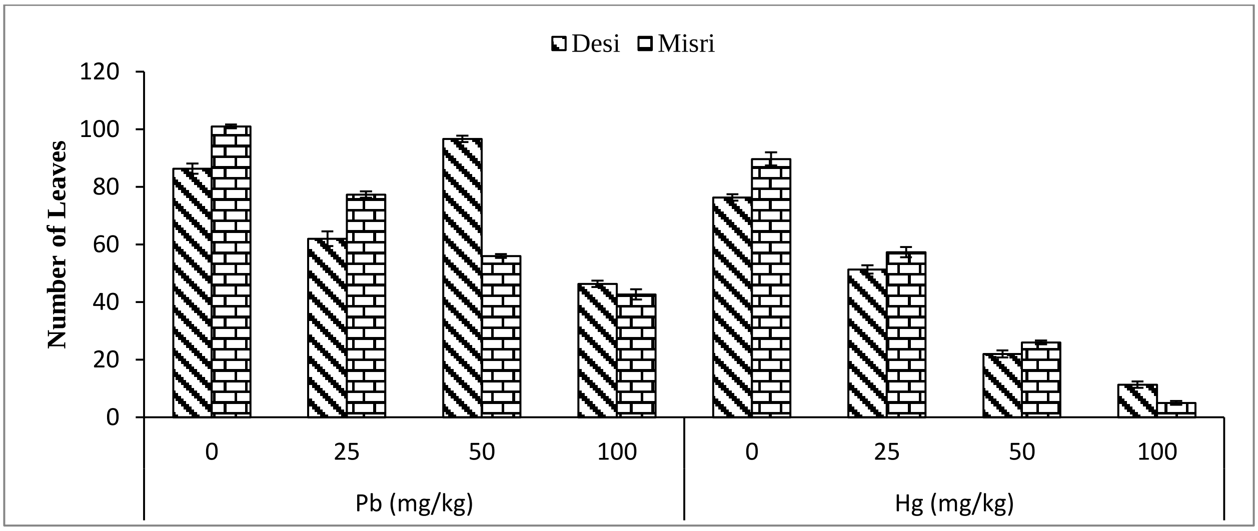

Fig. 8: Number of Leaves of Desi and Misri Varieties of *Trifolium alexandrinum* after Exposure to Control and Different Levels of Pb and Hg.

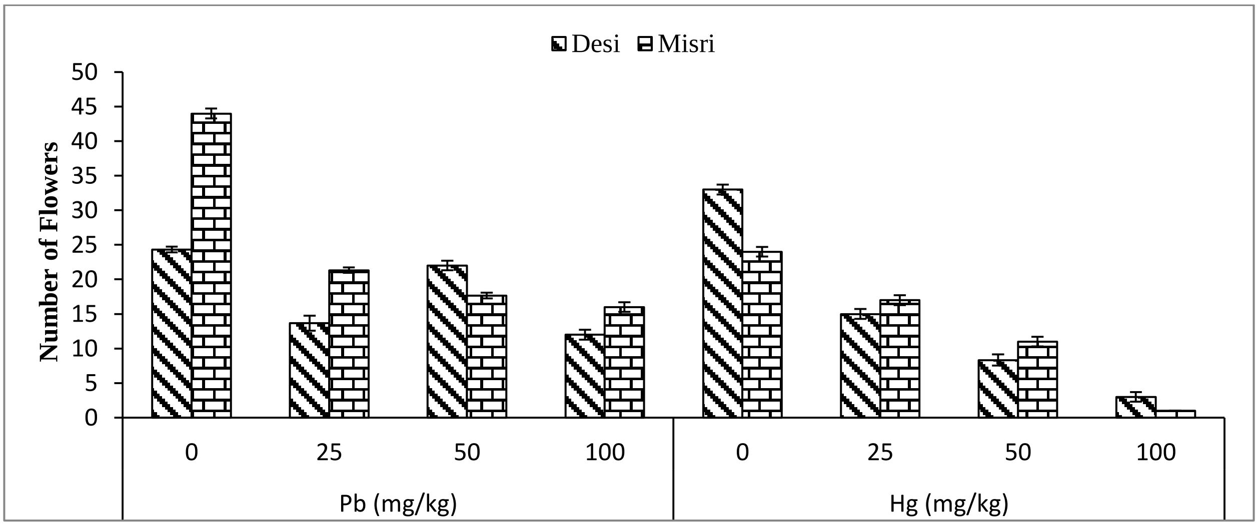

Fig. 9: Number of Flowers of Desi and Misri Varieties of *Trifolium alexandrinum* after Exposure to Control and Different Levels of Pb and Hg.

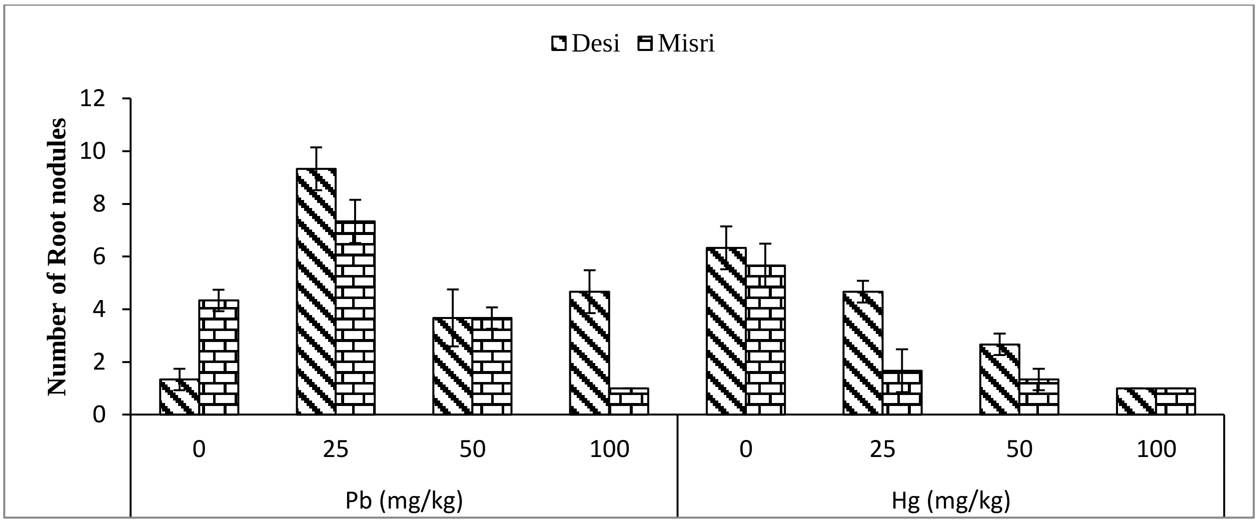

Fig. 10: Number of Root Nodules of Desi and Misri Varieties of *Trifolium alexandrinum* after Exposure to Control and Different Levels of Pb and Hg.

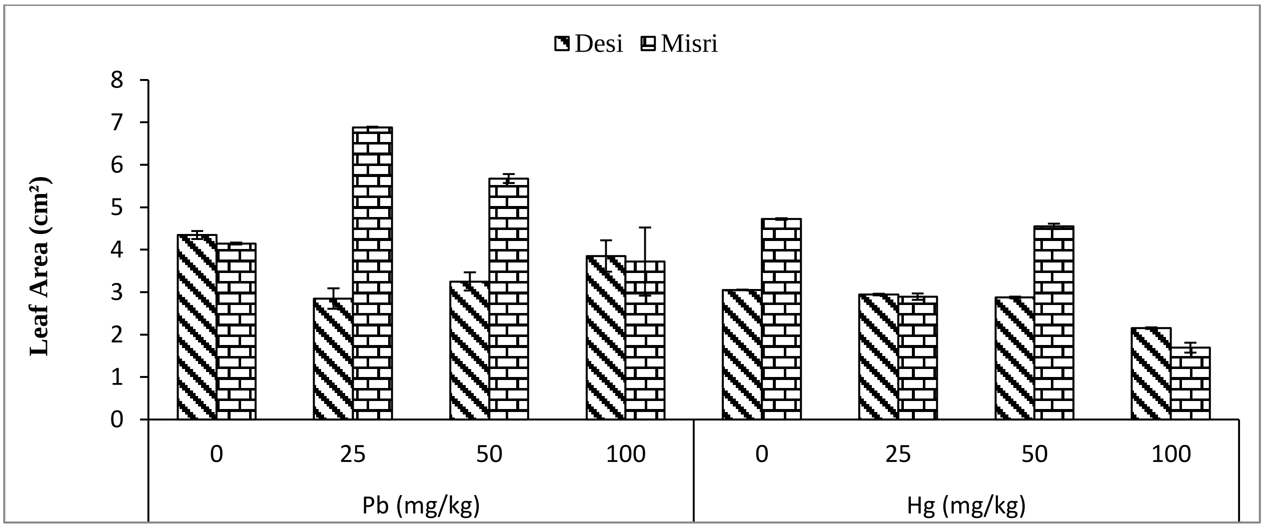

Fig. 11: Leaf Area (Cm^2) of Desi and Misri varieties of *Trifolium alexandrinum* after Exposure to Control and Different Levels of Pb and Hg.

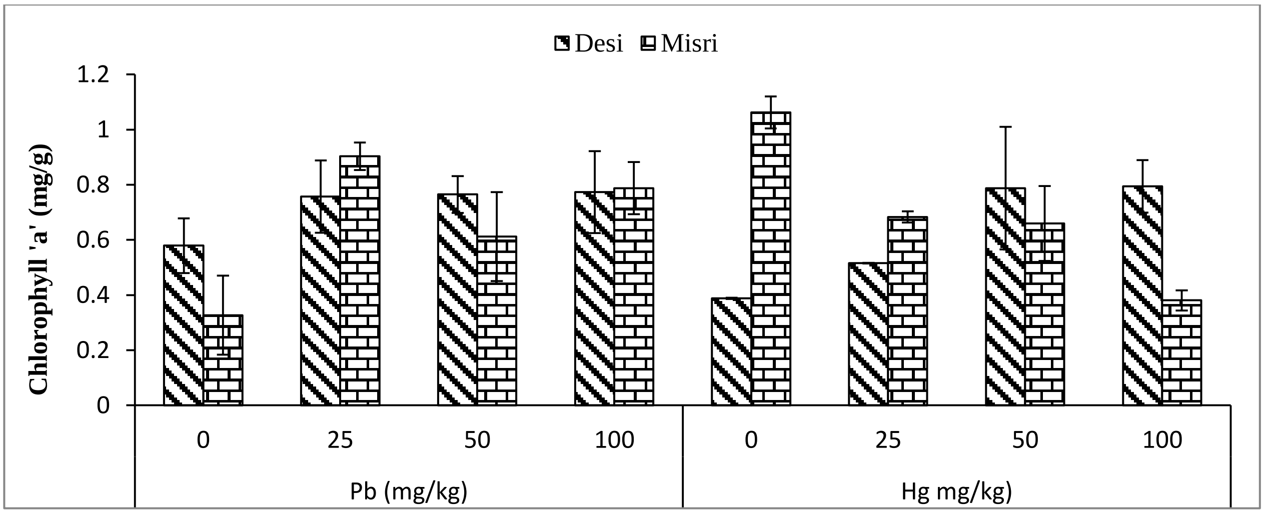

Fig. 12: Chlorophyll a (mg/g) of Desi and Misri varieties of *Trifolium alexandrinum* after Exposure to Control and Different Levels of Pb and Hg.

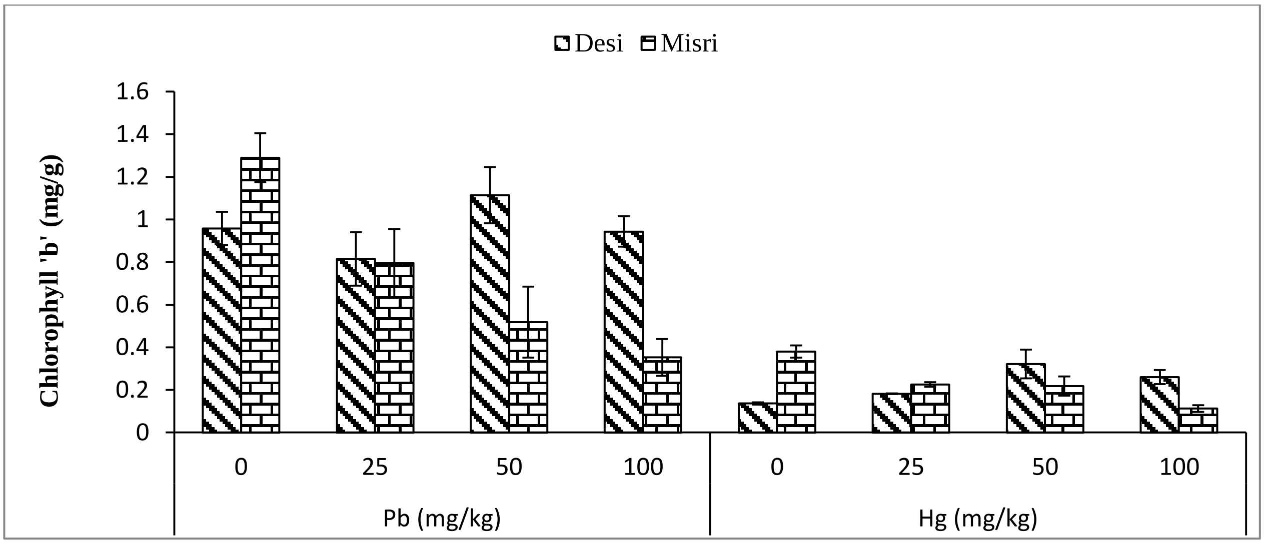

Fig. 13: Chlorophyll b (mg/g) of Desi and Misri varieties of *Trifolium alexandrinum* after Exposure to Control and Different Levels of Pb and Hg.

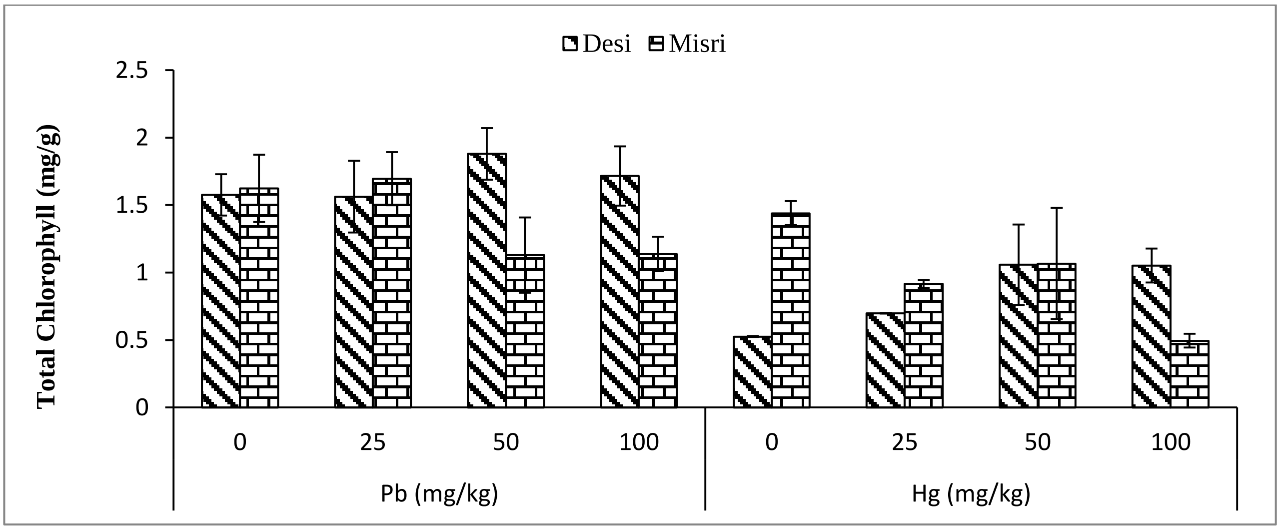

Fig. 14: Total Chlorophyll (mg/g) of Desi and Misri varieties of *Trifolium alexandrinum* after Exposure to Control and Different Levels of Pb and Hg.

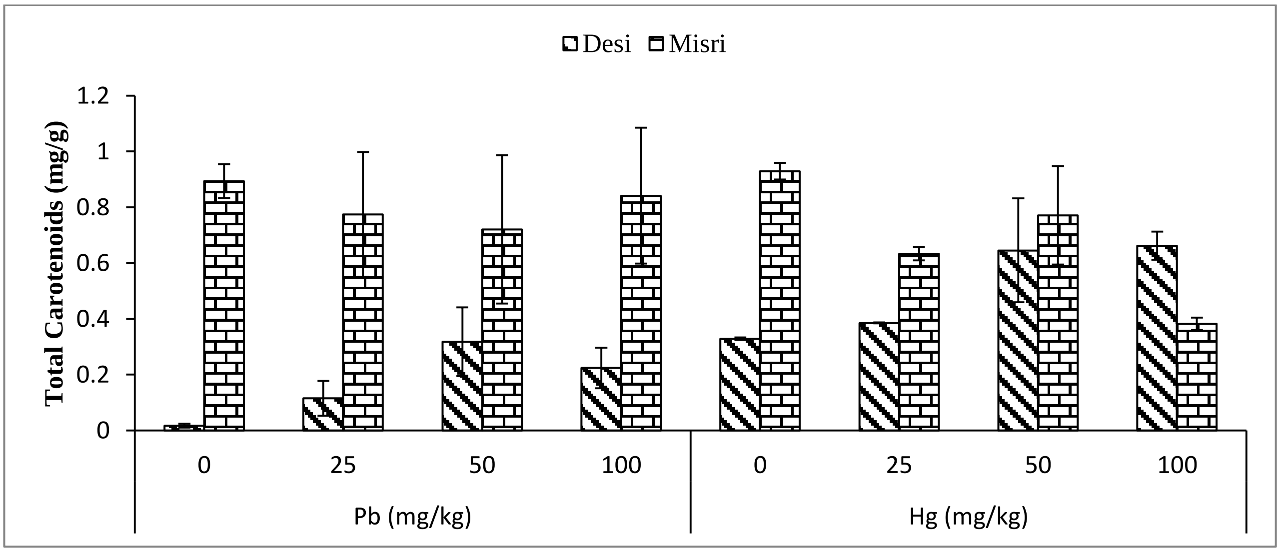

Fig. 15: Total Carotenoids (mg/g) of Desi and Misri varieties of *Trifolium alexandrinum* after Exposure to Control and Different Levels of Pb and Hg.

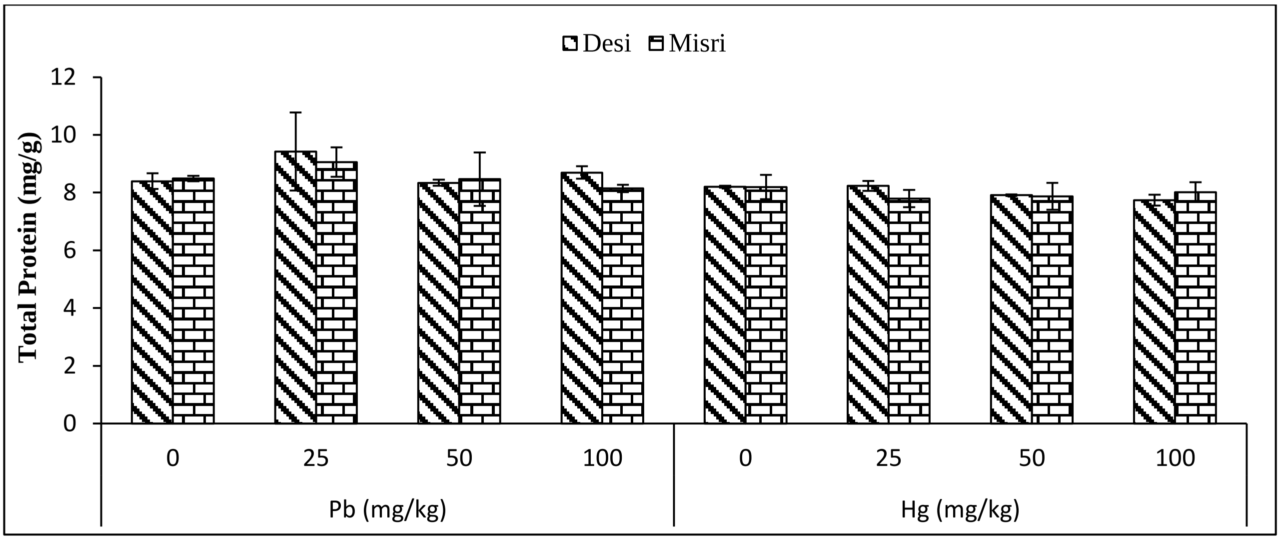

Fig. 16: Total Protein (mg/g) of Desi and Misri varieties of *Trifolium alexandrinum* after Exposure to Control and Different Levels of Pb and Hg.

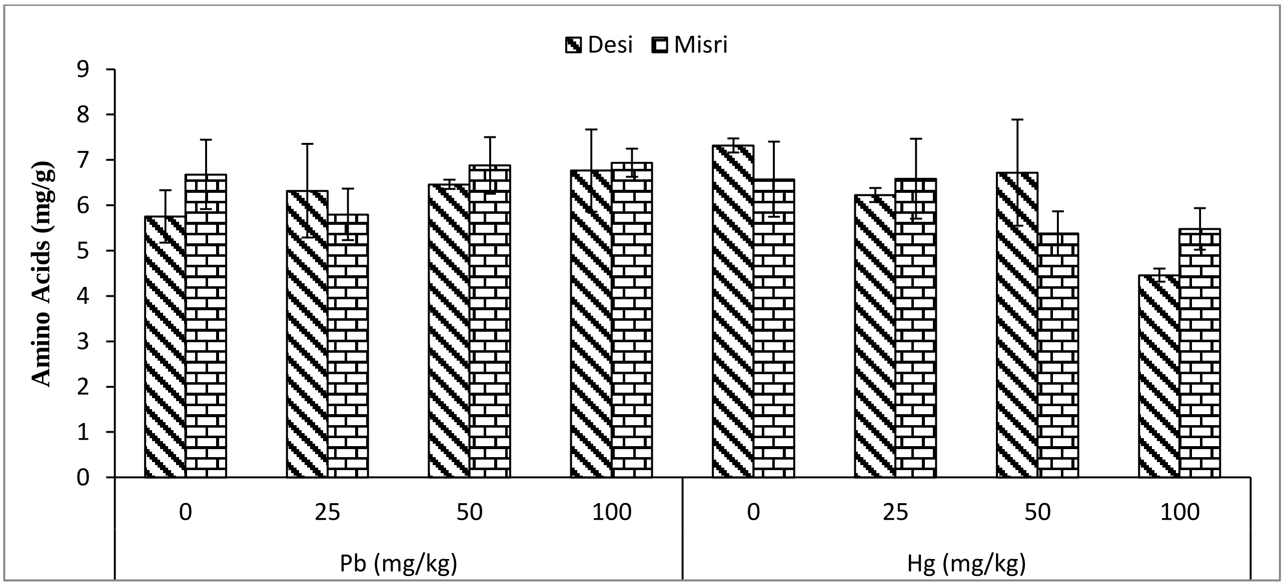

Fig. 17: Amino Acids (mg/g) of Desi and Misri varieties of *Trifolium alexandrinum* after Exposure to Control and Different Levels of Pb and Hg.

4. Conclusion

While summing up the work reported here it can be concluded that both varieties (Desi and Misri) of *Trifolium alexandrinum* L. showed considerably variable responses to both Pb and Hg.

The exposure of Pb and Hg at lower concentrations had no drastic effects on all parameters studied. However, the most elevated level of both metals caused more profound changes for growth and biometric parameters.

Misri performed better as compared to Desi as the former variety excelled for ten out of total seventeen parameters while the later showed tolerance for seven parameters in response to both Pb and Hg. Hg was found to be more toxic as compared to the Pb as it induced more drastic decline in almost all the biometric parameters studied.

Based on the results of the study it became evident that among biochemical attributes, pigments content did not depicted any marked influence thus could not be used as biochemical marker for the appraisal of metal tolerance in these varieties. However biometric traits can serve as useful predictors.

The study clearly signified that the two varieties of *T. alexandrium* had shown their potential for Pb and Hg contamination. Thus, the varieties can be grown under contaminated soil conditions or in situations where sewerage water is being in use for irrigation. However, screening of crop germ-plasm should be carried out on a large scale. Moreover, mechanistic explanation of Pb and Hg tolerance needs to be addressed in future studies.

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